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Role of the Proline-rich Domain of Dynamin-2 and Its Interactions with Src Homology 3 Domains during Endocytosis of the AT_1 Angiotensin Receptor*

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In nonneural tissues, the dynamin-2 isoform participates in the formation of clathrin-coated vesicles during receptor endocytosis. In this study, the mechanism of dynamin-2 action was explored during endocytosis of the G protein-coupled AT_{1A} angiotensin receptor expressed in Chinese hamster ovary cells. Dynamin-2 molecules with mutant pleckstrin homology domains or deleted proline-rich domains (PRD) exerted dominant negative inhibition on the endocytosis of radiolabeled angiotensin II. However, only the PRD mutation interfered with the localization of the dynamin-2 molecule to clathrin-coated pits and reduced the inhibitory effect of the GTPase-deficient K44A mutant dynamin-2. Green fluorescent protein-tagged Src homology 3 (SH3) domains of endophilin I and amphiphysin II, two major binding partners of dynamins, also inhibited AT_{1A} receptor-mediated endocytosis of angiotensin II. These effects were partially or fully, respectively, restored by the overexpression of dynamin-2. Transient overexpression of these SH3 domains also reduced the localization of dynamin-2 to clathrin-coated pits. These data indicate that, similar to the recruitment of dynamin-1 during the recycling of synaptic vesicles, interaction of the dynamin-2 PRD with SH3 domains of proteins such as the amphiphysins and endophilins is essential for AT_{1A} receptor endocytosis. This mechanism could be of general importance in dynamin-dependent endocytosis of other G protein-coupled receptors in nonneural tissues.

The AT_1 angiotensin receptor (AT_1-R)[1] is a member of the GPCR superfamily and mediates the physiological actions of the octapeptide hormone, Ang II, on cardiovascular regulation and salt/water balance. In rats and mice, the AT_1-R has functionally very similar AT_{1A} and AT_{1B} subtypes, which have greater than 90% sequence identity but different tissue distributions. Binding of Ang II to AT_1-Rs initiates conformational changes that lead to activation of their cognate G protein(s), predominantly $G_{q/11}$, in numerous Ang II target tissues. The receptors utilize a number of signaling pathways including stimulation of phospholipase C, causing Ca^{2+} signal generation and activation of protein kinase C isoforms and small G proteins, stimulation of receptor and nonreceptor tyrosine kinases, and activation of mitogen-activated protein kinases (1–3). In parallel with these signaling events, Ang II also causes rapid internalization of the AT_1-R (4, 5). Initial studies performed at saturating Ang II concentrations suggested that dynamin-independent mechanisms may mediate AT_1-R internalization (6). However, at physiological hormone concentrations the major mechanism of AT_1-R internalization is dynamin- and β-arrestin-dependent endocytosis via clathrin-coated vesicles (7–10).

Clathrin coat formation is thought to begin with recruitment of the AP2 clathrin adaptor complex to the plasma membrane, where it serves as a template for the assembly of the clathrin lattice. Studies on recycling of synaptic vesicles and internalization of nutrient and growth factor receptors have demonstrated that invagination of clathrin-coated pits and fission of clathrin-coated vesicles requires the recruitment of several accessory proteins, including dynamin, amphiphysin, endophilin, and synaptojanin (11–13). Dynamin is a 100-kDa GTPase that is thought to act at the fission step and is present in the cytosol as dimers or tetramers (14). During receptor endocytosis, dynamin assembles into ringlike structures around the neck of budding clathrin-coated pits, where it functions directly or indirectly in pinching off vesicles from the plasma membrane (15). Dynamin alone can polymerize into rings and can evaginate spherical liposomes into narrow tubules surrounded by polymerized dynamin (13, 16–19). However, other molecules interact with dynamin *in vivo* to facilitate its function and to assist in its recruitment to clathrin-coated pits (11, 12, 20).

Dynamin has several functional domains including an N-terminal GTPase domain, a PH domain, and a C-terminal proline-rich domain (PRD) (13, 15). The GTPase activity of dynamin is essential for its function, and GTPase-deficient mutants of dynamin act as dominant negative inhibitors of endocytosis (13, 15). The PH domain is a structural motif that is found in hundreds of proteins and has been shown to bind

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[1] The abbreviations used are: AT_1-R, type 1 angiotensin receptor; GPCR, G protein-coupled receptor; Ang II, angiotensin II; CHO, Chinese hamster ovary; dyn1, dynamin-1; dyn2, dynamin-2; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HA, influenza hemagglutinin; PH, pleckstrin homology; PRD, proline-rich domain; SH3, Src homology 3; SH3-Amph, SH3 domain of amphiphysin II; SH3-Endo, SH3 domain of endophilin I; SH3-Nck, N-terminal SH3 domain of Nck; GST, glutathione *S*-transferase.

with different preferences to various phophoinositides (21, 22). Certain PH domains that bind with high affinity and high specificity for phosphoinositides are capable of driving membrane recruitment (23). The PH domain of dynamin binds phosphoinositides with low affinity, and oligomerization of the molecule is required for strong binding of dynamin to inositol lipid-containing membranes *in vitro* (24). The importance of the PH domain in dynamin function is indicated by the ability of PH domain mutant dynamins with impaired phosphoinositide binding to act as dominant negative inhibitors of receptor endocytosis (25–27). These observations and some *in vitro* studies suggest that dynamin can be targeted to the plasma membrane during endocytosis through its PH domain (28). However, the PH domain also participates in the regulation of the GTPase activity of dynamin (13, 29).

The PRD of dynamin interacts *in vitro* with many SH3 domain-containing proteins, including proteins with established roles in endocytosis and recycling of synaptic vesicles (30). These endocytic proteins include amphiphysin and endophilin (13, 20, 31). Amphiphysin, which binds the clathrin heavy chain, the appendage domain of α-adaptin, dynamin, and synaptojanin, has been implicated in the recruitment of dynamin to clathrin-coated vesicles (11, 12). The SH3 domain of amphiphysin inhibits endocytosis in the lamprey reticulospinal synapse and in fibroblasts (31, 32). The SH3 domain-containing protein, endophilin, which also functions in endocytosis (33), has lysophosphatidic acid acyltransferase activity that appears to be essential for its function during the invagination of coated vesicles (34). However, endophilin also stimulates membrane vesiculation independent of this enzymatic activity (35). Although the major binding partner of endophilin is synaptojanin, a phosphatidylinositol 5-phosphatase, it also binds to the PRD of dynamin (11, 36). Previous studies have suggested that dynamin can be targeted to the membrane by interaction with SH3 domain-containing proteins (37–39).

Most of the data on dynamin's interaction with SH3 domain-containing proteins, such as amphiphysin and endophilin, was obtained using dyn1, the neuronal isoform of the molecule. These interactions are required for the recycling of synaptic vesicles via a clathrin-mediated process, and a similar process may operate during the endocytosis of hormone receptors in nonneural tissues. It is noteworthy that most endocytic proteins have neural and nonneural isoforms with very similar domain structures. In addition to its role in the formation of endocytic vesicles at the cell surface, dyn2, the ubiquitous isoform of dynamin, has been implicated in vesicle formation from endosomes and the Golgi (40, 41). Dynamins, particularly the nonneural dyn2 isoform, also appear to function as signaling molecules (42–45). Amphiphysins are predominantly expressed in neuronal tissues and bind to the same site on dynamin (PSRP*X*R) (46), but splice variants of amphiphysin II are also present in nonneuronal tissues (47). Although recent data have shown that the SH3 domains of endophilins 1 and 2 bind to the same motif (PP*X*RP), the tissue distribution of endophilin 2 suggests that it might be the partner of dyn2 in nonneuronal tissues (48, 49).

The role of dynamin during endocytosis of many GPCRs has been well established using GTPase-deficient dynamin mutants (13, 50, 51), but little is known about its mechanism of action in this process. In some GPCRs, including the AT_1-R, PH domain mutant dynamin has a dominant negative inhibitory effect on agonist-induced receptor endocytosis (7, 52). Endophilins directly interact with the β_1-adrenergic receptor and stimulate its agonist-induced internalization (53), but the role of their association with dynamin has not been demonstrated during the endocytosis of GPCRs. The role of the PRD in recruitment of dyn2 to clathrin-coated pits in nonneural tissues also has not been elucidated. The present study was performed to determine whether the role of ubiquitously expressed dyn2 isoform during agonist-induced endocytosis of the G protein-coupled AT_{1A} receptor in nonneuronal cells is analogous to that of the neuronal isoform of dynamin, dyn1, during the recycling of synaptic vesicles and endocytosis of nutrient and growth factor receptors.

EXPERIMENTAL PROCEDURES

Materials—The cDNA of the rat vascular smooth muscle AT_{1A} receptor was provided by Dr. K. E. Bernstein (Emory University, Atlanta, GA). The cDNAs of the HA epitope-tagged wild-type and K44A mutant dyn2 subcloned into pcDNA3 vector were kindly provided by Dr. K. Nakayama (Tsukuba Science City, Ibaraki, Japan). The GST-fused N-terminal SH3 domain of Nck was provided by Dr. László Buday (Semmelweis University, Budapest, Hungary) and was subcloned into pEGFP-C2 expression vector. GFP-β_2-adaptin was obtained from Dr. Marc G. Caron (Duke University Medical Center, Durham, NC). Anti-HA.11 monoclonal antibody was from Babco (Berkeley, CA), and horseradish peroxidase-conjugated goat anti-mouse antibody was from Pierce. Rhodamine-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Monoclonal antibody raised against GFP was a gift of Dr. László Buday. Unless otherwise stated, all other chemicals and reagents were from Sigma.

Plasmid Constructs, Mutagenesis, and Transfection—The SH3 domains of amphiphysin II and endophilin I with an N-terminal GFP tag were generated by amplification of the SH3 domain segment, which were subsequently cloned into the mammalian expression vector pEGFP-C2 as described earlier (39). Substitution of lysine 535 of dyn2 with alanine (dyn2-K535A) was performed using the Muta-Gene kit (Bio-Rad). Deletion of the PRD (dyn2-ΔPRD) was performed by the same method by introducing a stop codon in place of the proline 746 of dynamin. In the double mutants, dyn2-K44A/K535A and dyn2-K44A/ΔPRD, dyn2-K44A was used as template for generating the lysine 535 substitution with alanine or the PRD deletion, respectively. The sequences of the mutants were verified by dideoxy sequencing. CHO-K1 cells were transiently transfected in 24-well plates with plasmids containing AT_{1A}-R cDNAs and wild-type or mutant dynamins using 12 μg/ml LipofectAMINE (Invitrogen) as described previously (54). For confocal microscopy, cells were grown on glass coverslips and transfected with the indicated constructs using 3 μl/ml FuGENE 6 (Roche Diagnostics, Nutley, NJ). CHO cells were maintained in $NaHCO_3$-buffered Ham's F-12 medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin (Invitrogen).

Receptor Endocytosis in Transiently Transfected CHO Cells—To determine the internalization kinetics of the AT_{1A}-R, ^{125}I-Ang II (2.5 kBq/ml (~0.03 nM)) was added in 0.25 ml of HEPES-buffered Ham's F-12, and the cells were incubated at 37 °C for the indicated times. Incubations were stopped by placing the cells on ice and rapidly washing them twice with ice-cold phosphate-buffered saline. Acid-released and acid-resistant radioactivities were separated and measured by γ-spectrometry as described previously (54). The percentage of internalized ligand at each time point was calculated from the ratio of the acid-resistant specific binding to the total (acid-resistant + acid-released) specific binding.

Western Blot Analysis—For immunodetection of expressed proteins 48 h after transfection, cells were scraped into 200 μl of Laemmli buffer containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml trypsin-chymotrypsin inhibitor, 10 μg/ml pepstatin A, 10 μg/ml benzamidine). After centrifugation, the supernatant proteins were analyzed on 8% denaturing polyacrylamide gels and transferred to nitrocellulose membranes. Blots were then probed with primary antibody and detected with horseradish peroxidase-conjugated secondary antibodies using the SuperSignal West Pico detection kit (Pierce).

Immunofluorescence and Confocal Laser-scanning Microscopy—For immunofluorescence studies, CHO cells were grown on glass coverslips and transiently transfected as described above. 48 h later, the cells were washed twice with phosphate-buffered saline prior to fixation with 4% paraformaldehyde. The cells were then incubated with sodium borohydride (1 mg/ml) for 15 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. Incubation with anti-HA antibody (1:100) for 1 h at room temperature was followed by two 15-min washes with 25 mM Tris HCl (pH 7.4) in 0.14 M NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20 and incubation for 1 h with rhodamine-conjugated goat anti-mouse antibody (1:100). The coverslips were mounted using Dako Fluores-

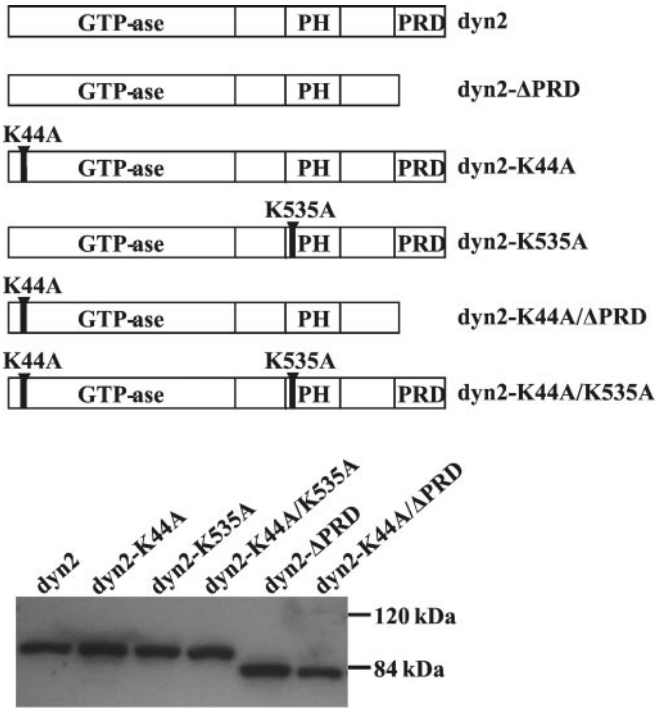

FIG. 1. **Mutant rat dyn2 constructs used in the present study.** *A*, the dyn2-ΔPRD is a C-terminal deletion mutant of dyn2 from position 746. The dyn2-K44A (40) and dyn2-K535A (7, 25–27) mutations affect the function of the GTPase domain and the PH domain, respectively. Double mutants, dyn2-K44A/ΔPRD and dyn2-K44A/K535A, are also shown. *B*, expression levels of wild-type and mutant dyn2 constructs were detected by Western blot analysis with a mouse monoclonal anti-HA antibody. The data are representative of three independent experiments.

cence mounting medium, and images were detected with a Zeiss LSM 510 confocal laser-scanning microscope. GFP and rhodamine were excited with argon (488-nm) and helium/neon (543-nm) lasers, and emitted fluorescences were detected in multitrack mode with 500–550- and 565–615-nm bandpass filters, respectively.

RESULTS

Dominant Negative Inhibition of AT_{1A}-R Endocytosis by PRD and PH Domain Mutants of dyn2—To study the role of the PRD of dyn2 during AT_1-R endocytosis, a dyn2 mutant was created (dyn2-ΔPRD), in which the entire C-terminal proline-rich domain was deleted commencing at proline 746 (Fig. 1*A*). When co-expressed with the AT_{1A}-R in CHO cells, this construct had a dominant negative inhibitory effect on the endocytosis of the receptor (Fig. 2*A*). The expression levels of the mutant dyn2 constructs in CHO cells are shown in Fig 1*B*. However, the extent of inhibition was smaller than that caused by the GTPase-deficient mutant, dyn2-K44A. To determine whether deletion of the dyn2 PRD affects the endocytosis of the AT_{1A}-R because it interferes with the localization of dyn2, a double mutant of dyn2 containing both the K44A replacement and the PRD deletion was created. It was expected that the ΔPRD mutation would reverse the dominant negative inhibitory effect of the K44A mutation if PRD deletion interferes with the localization of dyn2 to clathrin-coated vesicles. The inhibitory effect of the K44A/ΔPRD mutant dyn2 on AT_{1A}-R endocytosis was similar to that of the dyn2-ΔPRD, whereas it was considerably less than that of the K44A mutant (Fig. 2*A*). These data indicate that deletion of the PRD interferes with dynamin function by preventing its localization in clathrin-coated pits.

The role of the PRD in dynamin function was then compared with that of the PH domain. Substitutions of lysine 535 in the PH domain of dyn2 interfere with the phosphoinositide binding of the construct, and K535A and K535M dynamins have been

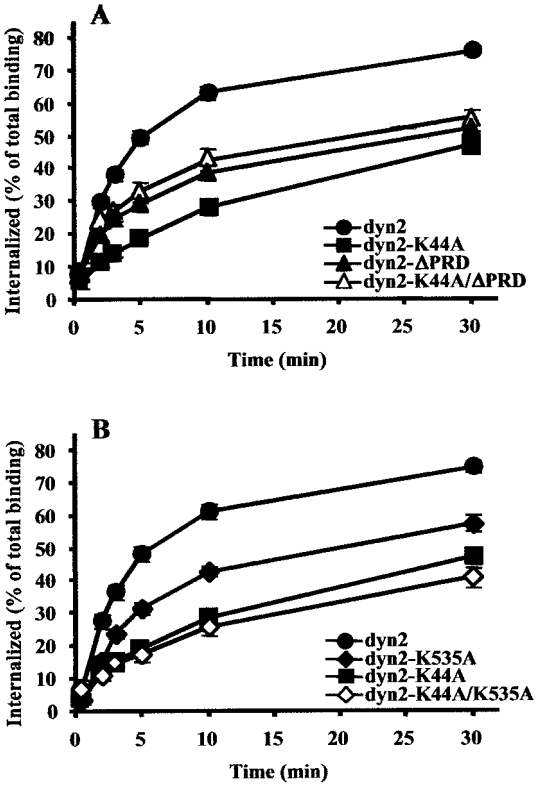

FIG. 2. **Effects of overexpressed dyn2 mutants on the kinetics of AT_{1A}-R endocytosis in CHO cells.** *A*, cells were grown in 24-well plates and co-transfected with 0.5 μg of AT_{1A}-R cDNA and 0.5 μg of wild-type dyn2 (●), dyn2-ΔPRD (▲), dyn2-K44A (■), and dyn2-K44A/ΔPRD (△). Endocytosis of ^{125}I-Ang II was measured as described under "Experimental Procedures." Data are shown as means ± S.E. of four independent experiments, each performed in duplicate. *B*, endocytosis of ^{125}I-Ang II in CHO cells co-transfected with 0.5 μg of AT_{1A}-R cDNA and 0.5 μg of wild-type dyn2 (●), dyn2-K535A (♦), dyn2-K44A (■), or dyn2-K44A/K535A (◇). Experimental conditions were the same as in *A*.

shown to exert dominant negative inhibitory effects on AT_1-R endocytosis (7, 52). Combination of the K535A and the K44A mutations increased the dominant negative inhibition of AT_1-R endocytosis compared with the individual dyn2-K535A mutation, and its effect was similar to that of the dyn2-K44A mutation (Fig. 2*B*). These data suggest that, unlike the PRD, the PH domain of dyn2 is not required for proper localization of the molecule during AT_1-R endocytosis.

Localization of the Mutant Forms of dyn2—The intracellular localization of transiently expressed dynamins in CHO cells was analyzed by immunostaining to detect the HA epitope tag attached to the wild-type and mutant dyn2 (40). Wild-type dyn2 showed a diffuse cytoplasmic pattern and also localized to the plasma membrane (Fig. 3*A*). Replacement of lysine 535 by alanine in the PH domain had no major effect on the localization of the mutant molecule as compared with wild-type dyn2 (Fig. 3*B*). As previously shown (40), dyn2-K44A appeared in punctate intracellular structures and also accumulated in larger structures associated with the cell membrane (Fig. 3*D*). In contrast, whereas the dyn2-ΔPRD mutant also appeared in punctate cytoplasmic structures, no membrane localization was observed (Fig. 3*C*). The localization of the dyn2-K44A/K535A (Fig. 3*E*) and dyn2-K44A/ΔPRD (Fig. 3*F*) double mutant constructs was not distinguishable from that of the dyn2-K44A and the dyn2-ΔPRD, respectively.

The presence of wild-type and mutant dyn2 molecules in clathrin-coated pits was determined based on co-localization with GFP-tagged β_2-adaptin (Fig. 4), an essential component of the AP2 clathrin adaptor protein (12, 55, 56). As previously

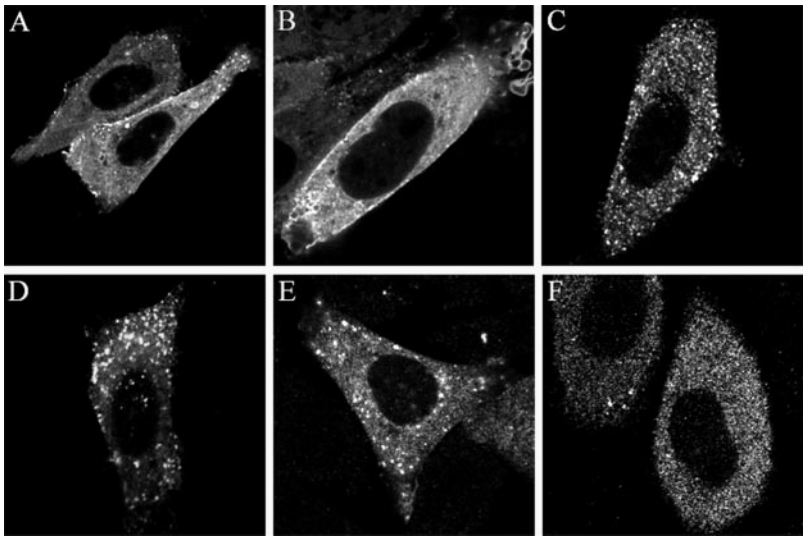

FIG. 3. **Subcellular localization of HA epitope-tagged dyn2 mutants transiently expressed in CHO cells.** CHO cells were co-transfected with a plasmid containing the AT_{1A}-R cDNA and dyn2 (*A*), dyn2-K535A (*B*), dyn2-ΔPRD (*C*), dyn2-K44A (*D*), dyn2-K44A/K535A (*E*), or dyn2-K44A/ΔPRD (*F*) as described under "Experimental Procedures." The cells were stimulated with Ang II (100 nmol/liter, 10 min, 37 °C) and stained with monoclonal anti-HA antibody as described under "Experimental Procedures." Typical cells are shown from a representative example of three experiments with identical results.

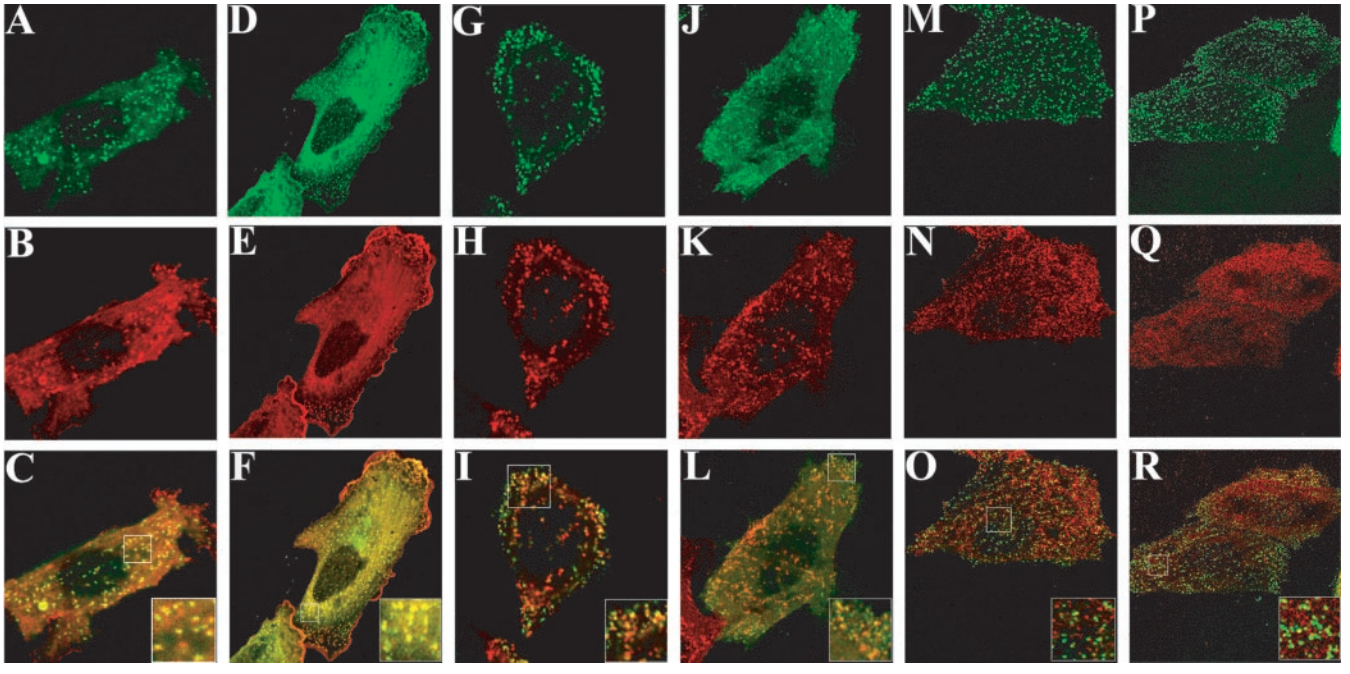

FIG. 4. **Co-localization of dyn2 mutants with GFP-β_2-adaptin in clathrin-coated pits.** CHO cells were transiently co-transfected with AT_{1A}-R, GFP-β_2-adaptin, and dyn2 (*A–C*), dyn2-K535A (*D–F*), dyn2-K44A (*G–I*), dyn2-K44A/K535A (*J–L*), dyn2-ΔPRD (*M–O*), or dyn2-K44A/ΔPRD (*P–R*). After stimulation with Ang II (100 nmol/liter, 10 min, 37 °C), the cells were fixed and stained with monoclonal anti-HA antibody and rhodamine-conjugated secondary antibody. The fluorescence of GFP-β_2-adaptin is shown in *green* in *A*, *D*, *G*, *J*, *M*, and *P*. Fluorescence of dyn2 (*B*), dyn2-K535A (*E*), dyn2-K44A (*H*), dyn2-K44A/K535A (*K*), dyn2-ΔPRD (*N*), or dyn2-K44A/ΔPRD (*Q*) is shown in *red*. The overlays are shown in *C*, *F*, *I*, *L*, *O*, and *R*. Typical cells are shown from a representative example of three experiments with identical results.

shown, β_2-adaptin-GFP was detectable in the cytosol and was co-localized with clathrin-coated pits (55). To detect enrichment of these structures at the plasma membrane, images were taken at the surface of the cell from the layer adjacent to the coverslip (Fig. 4). At the plasma membrane, both wild-type (Fig. 4*B*) and K535A (Fig. 4*E*) mutant dyn2 showed extensive co-localization (Fig. 4, *C* and *F*) with the β_2-adaptin-GFP labeling clathrin-coated pits (Fig. 4, *A* and *D*). The K44A mutant dyn2 (Fig. 4*H*) likewise showed co-localization with β_2-adaptin-GFP at the cell surface (Fig. 3*I*) but also appeared in clathrin-independent structures, a finding consistent with its occurrence in punctate intracellular structures (Fig. 4*D*) as described above. The double mutant dyn2-K44A/K535A (Fig. 4*K*) showed a similar partial co-localization with β_2-adaptin-GFP (Fig. 4*L*). However, Fig. 4, *O* and *R*, show that no significant co-localization of dyn2-ΔPRD (Fig. 4*N*) and dyn2-K44A/ΔPRD (Fig. 4*Q*) was observed with β_2-adaptin-GFP (Fig. 4, *M* and *P*), suggesting that this construct is not recruited to clathrin-coated vesicles. These data suggest that the PRD is required for recruitment of dyn2 to clathrin-coated pits at the plasma membrane and that the PH domain of the molecule has no significant role in this process.

Effects of Amphiphysin and Endophilin SH3 Domains on AT_{1A}-R Endocytosis—The role of the PRD of dyn2 during AT_{1A}-R endocytosis was also investigated by studying the effects of its potential binding partners on this process. As detailed above, earlier studies have established that amphiphysin and endophilin can interact with separate regions of the PRD of dyn2 via their SH3 domains. GFP-tagged versions of the SH3-Endo and SH3-Amph were co-expressed with the AT_{1A}-R to study the effects of these constructs on the internalization kinetics of the receptor. The expression levels of these GFP-

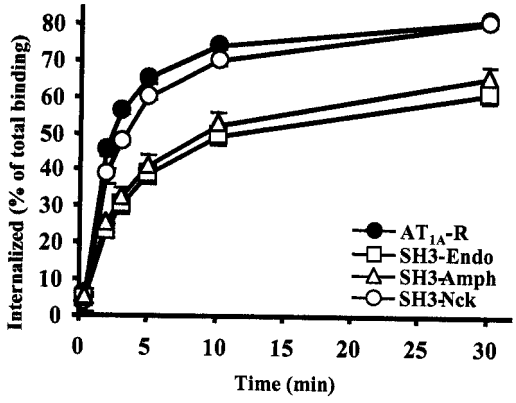

FIG. 5. **Effects of overexpressed GFP-SH3 domains of endophilin, amphiphysin, and Nck on the kinetics of AT_{1A}-R endocytosis.** CHO cells were grown in 24-well plates and co-transfected with 0.5 μg of AT_{1A}–R cDNA-containing plasmid (●) and 0.5 μg of GFP-tagged SH3-Endo (□), GFP-tagged SH3-Amph (△), or GFP-tagged SH3-Nck (○). Endocytosis of ^{125}I-Ang II was measured as described under "Experimental Procedures." Data are shown as means ± S.E. of seven independent experiments, each performed in duplicate.

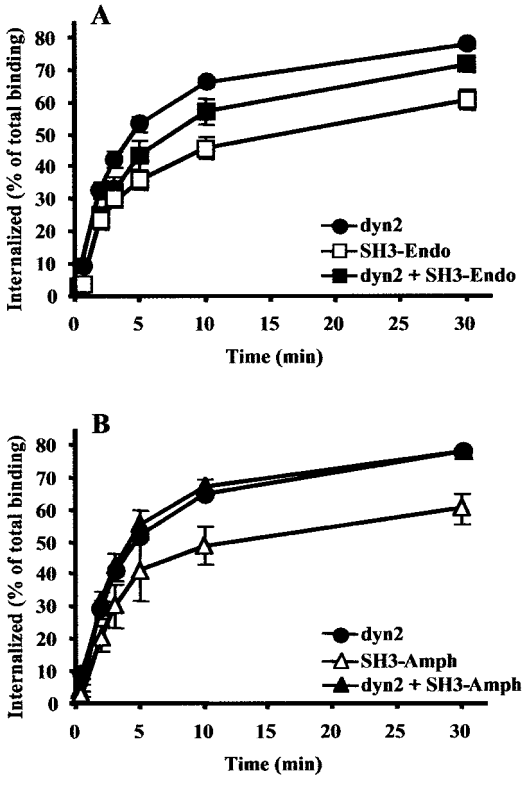

FIG. 6. **Overexpression of dyn2 interferes with the inhibitory effects of endophilin I and amphiphysin II SH3 domains on AT_{1A}-R endocytosis.** *A*, CHO cells were grown in 24-well plates and co-transfected with 0.5 μg of AT_{1A}-R cDNA and 0.5 μg of dyn2 (●), 0.5 μg GFP-tagged SH3-Endo (□), or 0.5 μg GFP-tagged SH3-Endo and 0.5 μg dyn2 (■). *B*, CHO cells were grown in 24-well plates and cotransfected with 0.5 μg of AT_{1A}-R cDNA and 0.5 μg of dyn2 (●), 0.5 μg of SH3-Amph (△), or 0.5 μg SH3-Amph and 0.5 μg of dyn2 (▲). Endocytosis of ^{125}I-Ang II was measured as described under "Experimental Procedures." Data are shown as means ± S.E. of three independent experiments, each performed in duplicate.

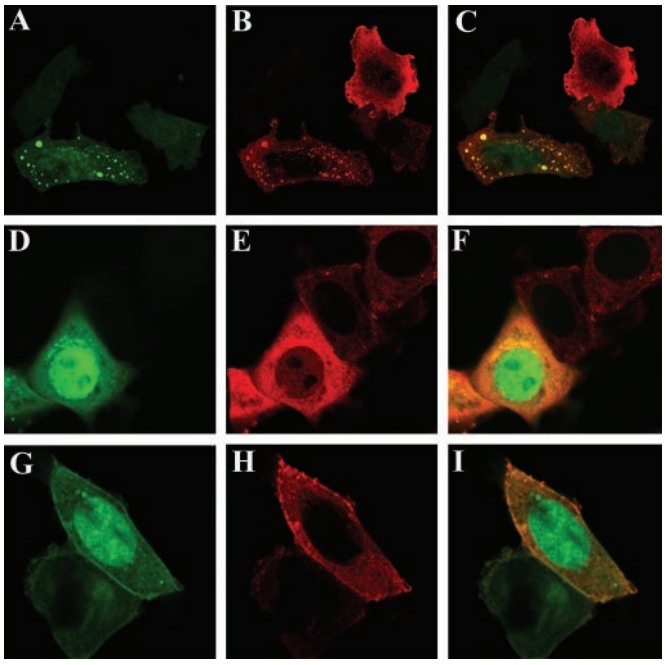

FIG. 7. **Subcellular localization of co-transfected HA epitope-tagged dyn2 and the GFP-tagged SH3-Endo, SH3-Amph, and SH3-Nck.** CHO cells were co-transfected with 0.5 μg of AT_{1A}-R cDNA and 0.5 μg of HA-dyn2 in combination with either 0.5 μg of GFP-tagged SH3-Endo (*A–C*), 0.5 μg of GFP-tagged SH3-Amph (*D–F*), or 0.5 μg of GFP-tagged SH3-Nck (*G–I*). After stimulation with Ang II (100 nmol/liter, 10 min, 37 °C), the cells were fixed and stained with monoclonal anti-HA antibody. The fluorescence of GFP-tagged SH3-Endo (*A*), SH3-Amph (*D*), and SH3-Nck (*G*) are shown in *green*. Localization of HA-dyn2 (*B*, *E*, and *H*) is shown in *red*. Overlays are shown in *C*, *F*, and *I*. In the *top panels* (*A–C*), a cell with no detectable expression of GFP-tagged SH3-Endo (*top right corner*) shows typical plasma membrane and cytoplasmic localization of dyn2, and another cell with high expression of GFP-tagged SH3-Endo (*lower left corner*) shows localization of SH3-Endo and dyn2 to punctate cytosolic structures with loss of dyn2 from the plasma membrane. In the *middle panels* (*D–F*), cells with no detectable expression of GFP-tagged SH3-Amph (*top right*) show typical localization of dyn2. In cells that express high levels of SH3-Amph, the plasma membrane localization of dyn2 is not detectable. In the *lower panels* (*G* and *H*), the cell in the middle expresses GFP-tagged SH3-Nck, which does not bind to dynamin, and the characteristic plasma membrane localization of dyn2 is not affected. Typical cells are shown from a representative example of three experiments with identical results.

tagged constructs were monitored by Western blotting with a GFP-specific antibody (data not shown). Co-expression of both SH3 domains exerted dominant negative inhibitory effects on the endocytosis of the receptor (Fig. 5). In contrast, GFP alone (data not shown) or SH3-Nck (Fig. 5), which does not bind to dynamin *in vitro* (57), did not affect the endocytosis of the receptor. The inhibitory actions of SH3-Endo and SH3-Amph suggest that interaction of dyn2 with these proteins is required during AT_{1A}-R endocytosis. To determine whether these SH3 domains exerted their inhibitory effect by blocking the physiological interactions of dyn2's PRD, the effect of overexpression of dyn2 was studied. Expression of wild-type dyn2 had a minor inhibitory effect on AT_{1A}-R endocytosis (7). However, co-expression of dyn2 with the SH3-Endo (Fig 6*A*) or SH3-Amph (Fig. 6*B*) partially or fully, respectively, rescued the inhibitory effect of these constructs on AT_{1A}-R endocytosis. These data suggest that the interaction of the dyn2-PRD with endophilin and amphiphysin has a role in the recruitment of dynamin to clathrin-coated pits during AT_{1A}-R internalization.

Mislocalization of dyn2 in Cells Expressing SH3-Amph and SH3-Endo—The intracellular distribution of dyn2 was markedly affected in cells expressing the SH3 domain of endophilin compared with those expressing only dyn2 (Fig. 7, *A–C*). The plasma membrane localization and the even cytosolic distribution were lost, and the dynamin staining became punctate and was co-localized with the GFP-tagged SH3-Endo (Fig. 7, *A–C*). The plasma membrane localization of dyn2 was also lost in cells

expressing SH3-Amph (Fig. 7, *D–F*), but in this case the localization of dyn2 in the cytoplasm was homogenous (Fig. 7*E*). GFP-tagged SH3-Nck was detectable in the cytoplasm and showed homogenous localization to the plasma membrane without accumulation in clathrin-coated pits (Fig. 7*G*). Coexpression of GFP-SH3-Nck did not interfere with the localization of dyn2 (Fig. 7, *G–I*).

DISCUSSION

The essential role of dynamin during the formation of clathrin-coated vesicles is well established (12, 13, 15). In addition to its action in the scission of coated vesicles, dynamin has been proposed to have a role in the invagination of clathrin-coated pits (20). As with the recycling of synaptic vesicles and the endocytosis of nutrient and growth factor receptors, numerous GPCRs internalize via a dynamin-dependent mechanism (5, 50, 51, 58, 59). Most studies on this subject have examined the role of dyn1, but many GPCRs operate in nonneuronal tissues that express the ubiquitous dyn2 isoform (60). However, there are few data on the recruitment of dyn2 to clathrin-coated pits during the endocytosis of GPCRs.

In the present experiments, kinetic analysis of AT_{1A}-R endocytosis was performed at subnanomolar Ang II concentrations, because under these conditions the internalization of the receptor is mediated predominantly by dynamin-dependent endocytosis via clathrin-coated vesicles (7). Our observations demonstrate that the interaction of the PRD of dyn2 with SH3 domain-containing proteins is required for dynamin-dependent endocytosis of the AT_{1A}-R molecule. Deletion of the PRD of dyn2 or overexpression of the SH3 domains of endocytic proteins such as endophilin and amphiphysin interfered with the recruitment of dyn2 to clathrin-coated pits and exerted dominant negative inhibitory effects on AT_{1A}-R endocytosis. The dominant negative inhibitory effect of dyn2-ΔPRD is unusual, because most expressed proteins with mutations that cause mistargeting of the molecule do not interfere with the function of the normal endogenous protein. However, dynamin exists in the cytosol as tetramers (or oligomers) before clathrin coat assembly (61), and it appears that the presence of the mutant protein in these complexes interferes with the function of the normal endogenous molecule. The role of the PRD in localization of dyn2 is also consistent with the finding that deletion of the PRD reduces the inhibitory effect of dyn2-K44A on AT_{1A}-R endocytosis to that of ΔPRD-dyn2. An earlier study found that deletion of the PRD of dyn1 does not eliminate the inhibitory effect of the K44A mutation on transferrin receptor endocytosis (38). However, the inhibitory effect of the PRD-deleted dynamin on receptor endocytosis may explain these findings.

Co-localization of amphiphysin and endophilin with dynamin at the neck of coated pits in synaptic vesicles has been shown previously (62, 63). In the present study, expression of the SH3-Amph and SH3-Endo also inhibits endocytosis of the receptor and interferes with the localization of dynamin to clathrin-coated pits. Although expression of SH3 domains may interact nonspecifically with many different processes that involve proline-rich domains, it was shown that inhibition of receptor endocytosis with SH3 domains of endocytic proteins is fairly specific (31, 39). Furthermore, in the present study, the inhibitory effects of SH3-Amph and SH3-Endo were attenuated by overexpression of dyn2, indicating that they act by blocking interactions of the PRD of endogenous dyn2. These SH3 domains also interfered with the plasma membrane localization of dyn2. Amphiphysin has been proposed to participate in the recruitment of dynamin to clathrin-coated pits, because it binds to the clathrin heavy chain, the appendage domain of α-adaptin, dynamin, and synaptojanin (12, 47). The present data suggest that peripheral variants of amphiphysin II play a similar role in nonneuronal tissues. It has been reported recently that the SH3 domain of endophilin inhibits both late stages of invagination and scission of clathrin-coated vesicles *in vitro* by reducing phosphoinositide levels and interfering with the recruitment of the components of clathrin-coated pits to the plasma membrane (20). Although these mechanisms may operate under our experimental conditions, the present data suggest that endophilin also has a more specific role in dynamin recruitment because overexpression of dyn2 partially interfered with the inhibitory effect of SH3-Endo on AT_{1A}-R endocytosis. The importance of endophilin in dynamin recruitment is underlined by the recent identification of the binding site for endophilin SH3 domains on dynamin (PP*X*RP), which is present in a region of dynamin that was previously found to be the major determinant of dynamin localization to clathrin-coated pits (37, 48).

As shown earlier, a PH domain mutant dyn2 (dyn2-K535A) with reduced phosphoinositide binding also had a dominant negative inhibitory effect upon endocytosis of the AT_{1A}-R. Although the interaction of PH domains with phosphoinositides is generally believed to be crucial for the subcellular localization of the molecule (21, 64), and PH domains are widely used to map the localization of their lipid-binding partners (23), the K535A mutation did not interfere with the localization of the molecule to clathrin-coated pits. These data indicate that the PH domain of dynamin does not act as a subcellular localization signal but does have a functional role during dynamin action. These data are consistent with earlier findings that interaction of the PH domain of dynamin with lipids increases its GTPase activity (13). This mechanism could explain the additivity of the K535A mutation with the K44A mutation, since the latter change did not cause complete inhibition of the GTPase activity of the molecule (52).

In summary, the present data demonstrate that a dyn2 mutant with deletion of the PRD and the SH3 domains of amphiphysin II and endophilin I exerts dominant negative inhibitory effects on endocytosis of the G protein-coupled AT_{1A}-R. Confocal analysis of the localization of mutant and wild-type dyn2 and the GFP-tagged SH3 domains demonstrated that this interaction, but not the intact PH domain, is required for proper localization of the dyn2 molecule in clathrin-coated pits. These findings suggest that the actions of nonneural forms of dynamin, amphiphysin, and endophilin during dynamin-dependent endocytosis of a GPCR are similar to those described for the neuronal isoforms of these proteins.

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